A BIOMETRICAL STUDY OF THE MUCOSUS CAPSULATUS GROUP *

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For some time work has been carried on in an endeavor to decide if the micro-organisms now classed under the general head of mucosus capsulatus could be divided into distinct species after a biometrical review of their various characters.

So much work has been done in this group and the literature so thoroughly reviewed that only the more complete of the earlier publications need be mentioned. Fricke,[1] in 1896, gave the general characters of the group as follows: short, non-spore bearing, encapsulated bacilli, non-motile and gram-negative, showing a slimy growth on different media, not liquefying gelatin, sometimes forming indol, and fermenting certain of the carbohydrates. Clairmont,[2] in 1902, continued the study of the members of this group and in his work included reference to the reaction of agglutination, which however he found unsatisfactory as a means of differentiation. Perkins,[3] in 1904, proposed a simple classification based on the fermentation characteristics of these micro-organisms. The first species was designated as the bacterium aërogenes (including all strains of the bacilli lactis aërogenes, capsulatus septicus, Pfeiffer and Howard, and a large series isolated by Perkins. This group fermented the monosaccharids — dextrose and levulose; the disaccharids — saccharose, lactose and maltose, the pentose arabinose, the triatomic alcohol glycerin, and the hexatomic alcohol mannite. The second species, the bacterium pneumonicum, included all the Friedländer group, the bacillus ozenae, probably the bacillus rhinoscleromatis, and fermented all carbohydrates except lactose. The third species had as its prototype the bacillus acidi lactici, and members of this species fermented all the carbohydrates enumerated, with the exception of saccharose.

There has been no recent attempt made to classify this group on the basis of cultural features or biochemical activities alone. Behan,[4] however, has quite recently endeavored to differentiate these various species by means of the reaction of agglutination, with apparently some success.

Our study was undertaken in the hope that the biometrical methods employed by Winslow in the study of the coccaceae, might serve to finally elucidate the problem as to whether or not micro-organisms represent one or more species which were heretofore described as belonging to many species.

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1. *Ztschr. f. Hyg. u. Infectionskrankh.*, 1896, 23, p. 380.
2. *Ibid.*, 1902, 39, p. 1.
3. *Jour. Infect. Dis.*, 1904, 1, p. 241.
4. *Centralbl. f. Bakteriol.*, Abt. 1, Orig., 1912, 66, p. 110.

The earliest description of many of the bacteria investigated by us are utterly inadequate for the differentiation of species, for examples, Fricke's[5] endeavor to separate the species on the basis of differences in the appearance on potato, and Clairmont's[6] on the basis of the time of coagulation of milk. The later work of Perkins[7] and of Strong[8] showed the weaknesses of such criteria. Perkins, as a result of his study of this group, investigated thirty-seven strains.

This classification of Perkins assumes constant results from a study of the fermentation reactions of these species. Beham's[9] later work is an endeavor to show that the immunity reactions, the agglutination reaction in particular, is probably of greater value in the differentiation than any cultural features or biochemical reactions. It seemed that the most satisfactory results would be obtained if a very considerable number of micro-organisms, from widely different sources, were studied in a fashion that would show: (1) the common characteristics of all the members of the group, and (2) an analysis of differences on which it might be possible to group these bacteria in various species.

In all, forty-four cultures have been studied. These have been obtained from the Král collection, from the Pasteur Institute, Paris, from the Lister Institute, London, The American Museum of Natural History, Harvard Medical School, University of Chicago, Leland Stanford Junior University, Massachusetts General Hospital, University of California, The Cutter Laboratory, University of California Hospital, and the Infirmary, University of California. Fourteen of the forty-four cultures were designated as the bacterium capsulatus mucosus, Fasching; five cultures as the bacterium capsulatus Pfeiffer; six cultures as the bacillus aërogenes Escherich; four cultures as the bacterium ozenae Abel-Löwenberg; seven as the bacillus pneumoniae Friedländer, five as the bacterium rhinoscleromatis v. Frisch, and one as the bacterium crassus sputigenus Kreiböhm. To further round out the group two strains of the bacterium enteritidis Gaertner, were also included.

Obviously there are decided limitations in the conclusions to be drawn from a biometrical study of so few strains but an investigation of the characteristics on which species differentiation has been based has been of very considerable interest.

The following characteristics of these forty-four cultures have been studied in this work: (1) morphology, including a study of form, arrangement, capsule formation on different media, and motility· (2) staining reactions, and here the relation of this group to the bactericidal or bacteriostatic effect of gentian violet has been investigated, as well as the gram-staining reaction of the group; (3) cul-

5. *Ztschr. f. Hyg. u. Infectionskrankh.*, 1890, 23, p. 380.
6. *Ibid.*, 1902, 39, p. 1.
7. *Jour. Infect. Dis.*, 1904, 1, p. 241.
8. *Centralbl. f. Bakteriol.*, Abt. 1, Orig., 1889, 25, p. 49.
9. *Ibid.*, 1912, 66, p. 110.

tural features and biochemical reactions including also a study of the question of the production of a diffusible toxin by certain of the gram-negative, encapsulated bacilli; (4) the immunity or specific serum reactions; and (5) the pathogenicity.

It is hardly to be expected that results of very great value from the point of view of differentiation were to be gained by a study of form alone. An analysis of the results of smears examined shows that the members of this group vary in form from cocco-bacilli to long rods not the least suggesting the coccus-like forms. The length of the bacilli seen in different cultures varied greatly, and cultures with the same designation, from different sources, showed extremely wide variations in morphology. This was perhaps the most conspicuous feature. The cocco-bacilli were found amongst cultures of the so-called bacilli rhinoscleromatis, pneumoniae Friedländer, and capsulatus mucosus. There was no uniformity in this, however, and curves do not show any characteristic mode for any group of these cultures. The arrangement of the bacilli did not tend to show any constant feature, such as the parallel arrangement seen in cultures of the diphtheria bacillus. All the cultures here included presented capsules at some time or other. A few had lost the capsule between the time of isolation and the beginning of this work. Some of these cultures were made to regain their capsule by passage through animals.

All studies of morphology and capsule formation were undertaken on agar cultures after twenty-four hours at 37 C., for form, and on agar and blood serum cultures, for capsule formation. The methods of Hiss, Rosenow and Buerger for demonstrating capsules were utilized. All of these methods gave very satisfactory results. All micro-organisms which were included in this group were encapsulated bacilli or cocco-bacilli, though the facility with which the capsule is lost varies greatly in different cultures. So far as the characteristics already studied are concerned, all of these micro-organisms could very well be included under one species. None of the forty-four cultures here studied was motile.

The staining reactions were next analyzed. Though the point was made by Fricke, and by later workers, including Perkins, Strong and others, the Gram staining characteristics of these bacteria are still used to distinguish the ozena bacillus and the rhinoscleroma bacillus, as I have elsewhere pointed out. McFarland[10] in his text-book, and

10. Pathogenic Bacteria, 1912, p. 785.

Page[11] in an article on the ozena bacillus, state that the rhinoscleroma bacillus is a gram-positive bacillus, and so can easily be differentiated from the ozena bacillus.

We have yet to find a culture, in this group, when stained by Gram's method showing the characteristics of a gram-positive micro-organism, and it is probable that, unless one is dealing with a gram-negative encapsulated bacillus, it does not belong to this group. This point cannot be too strongly insisted on as the present great confusion, with reference to the group under discussion, is in part a result of species differentiation based on insufficient data. With the study of the gram-staining properties of the group, the influence in the growth of the cultures of solution of gentian violet on agar, and on serum-agar plate cultures, was observed. Here again, the result was interesting. Of 3 cultures of the ozena bacillus, the growth of 1 was influenced, while 2 were not affected; with 5 cultures of the bacillus pneumoniae, 2 were inhibited, but 3 were not. Five of the 14 cultures of the bacillus capsulatus were inhibited, and 9 were not. None of the rest of the 40 cultures were in any way modified in their growth in the presence of gentian violet solutions. In the three species, members of which were inhibited, there was no constancy shown, and further from 33.33 + to 40 percent of each group were so affected. Although the action of gentian violet, in inhibiting the growth of bacteria, has served to differentiate certain other closely related species it was of no value in this instance. In connection with this interesting action of gentian violet first described by Churchman,[12] we have found it to be of value in the isolation of gram-negative encapsulated bacilli. These micro-organisms, when found in certain situations, especially in the nose, are commonly found associated with various species of gram-positive cocci which tend to overgrow at first, the more delicate gram-negative bacilli. By the use of gentian violet serum-agar plates, we have been able to isolate these bacilli much more readily than by using the ordinary plating methods.

In studies of the cultural features of bacteria, much stress is often laid on differences of minor importance which really are of little value in differentiation. On agar slants, potato, and other media, members of the capsulatus mucosus group are believed to present a slimy mucoid growth. This is most variable. If the culture medium

11. *Jour. Med. Research*, 1912, 26, p. 489.
12. *Jour. Exper. Med.*, 1912, 16, p. 221.

is freshly prepared and contains an abundance of moisture this characteristic is in evidence, otherwise one does not frequently observe it.

The cultural characteristics on potato medium, agar, blood serum in broth, etc., did not present anything of value in differentiation. No previous workers had investigated a large series of these cultures to determine whether their biochemical activities (other than their action on carbohydrates) might be of value in showing differences in species, consequently we devoted considerable attention to this question. We have found that strains of the bacilli capsulatus mucosus, ozenae and pneumoniae Friedländer, all may show proteolytic enzyme activity, as evidenced in their ability to digest blood-serum. Here again, though nothing was learned which could be used to differentiate the species. Similarly the enzyme activity of various members of the group was studied in gelatin. Three cultures of the bacillus capsulatus mucosus and one of the bacillus rhinoscleromatis gave most marked evidence of ability to liquefy gelatin. Cultures which gave parallel results in gelatin were found, however, to differ in many essentials in their behavior on carbohydrates, so that their action on gelatin was apparently not a characteristic which could be utilized to arrange species groups. Only about 10 percent of the cultures of this group liquefied gelatin.

Twenty-three of forty-four cultures were shown to be capable of reducing nitrates to nitrites. Sulphanilic acid was used. Here again, there was no constant reaction according to designation of the cultures. Seventy-five percent of all the cultures were able to produce ammonia when tested with Nessler's reagent, but all species showed about an equal ability in this direction. In a series of tests of the Vosges-Proskaeur reaction, using tubes of 1 percent glucose peptone water, and later testing by adding potassium hydroxid solution, 8 of 43 cultures gave a positive reaction. Of the 8, 3 were labelled *B. capsulatus mucosus,* 3 *B. capsulatus* Pfeiffer, and 2 *B. aërogenes.*

All cultures were tested for indol production and all but eight gave evidence of ability to form this substance from the peptone molecule.

Here again, the cultures which did not form indol could not be grouped together when their action on carbohydrates was investigated, as no relation between these characteristics was found. Constant endeavor was made to parallel findings with any relations shown to exist as a result of the study of the fermentation reactions, because all the groupings so far suggested, with the exception of Beham's,

are based either on the action on carbohydrates, or carbohydrates and milk media.

Gèrmain claimed to have been able to differentiate closely related species by means of their ability to produce creatinin. Folin's and Solkowski's methods were employed for this purpose, and all cultures were tested for creatinin production. In all, eight cultures were shown to produce creatinin in 20 percent Dunham's solution after seven days, when tested by the colorimetric method using picric acid and 10 percent sodium hydrate and potassium bichromate solution as a standard, and also by using Weyl's sodium nitro-prusside method. Thus, while it was shown that various gram-negative encapsulated bacilli could produce creatinin, it was not possible to adduce evidence, which with other points elucidated, would serve to show that more than one species were concerned. As we analyze the evidence, on which earlier workers based their claim that we have several species amongst the gram-negative encapsulated bacilli, the results of the activity of our cultures in litmus milk are most instructive. Fricke found coagulation of milk with cultures of the bacillus pneumoniae Friedländer; Clairmont, Strong, and Perkins found no coagulation. In repeated tests with cultures of the bacillus pneumoniae Friedländer, from two different sources, one from the Pasteur Institute, Paris, and one from the American Museum of Natural History, one of these always failed to coagulate milk, while the other invariably produced coagulation. These results were obtained with chemically clean glassware. It would seem that any classification which emphasizes the point of differences in litmus milk, as an aid to the differentiation of species must be accepted with caution. Perkins has shown also that a culture of the bacillus capsulatus mucosus, which originally coagulated milk, did not do so when investigated by Strong and by himself. Furthermore, recent work has shown the fallacy of relying too much on fermentation tests since bacteria can be greatly modified in this particular, as Penfold has emphasized. Changes in litmus milk, ending in acid production and coagulation, are probably the result of lactose being broken up, consequently this test has about the same value as one of the fermentation tests.

These results with the bacillus pneumoniae Friedländer, so far as they relate to Strong's work and conclusions, are paralleled by findings with other members of the group, for example, with the bacillus rhinoscleromatis, where the same variation is found.

The next question, with reference to the classification of the members of this group, is the significance of their reactions in carbohydrate media. A classification based on one feature alone is of value when constant findings can be obtained. Obviously, if we are sure that all gram-negative encapsulated bacilli can be classified according to their biochemical reactions in carbohydrate media, the problem is greatly simplified. Therefore, we have endeavored to determine whether the fermentation tests may be regarded as satisfying the claims made for them. Strong in his work emphasized the value of three sugars; one monosaccharid-glucose, and two disaccharids—saccharose and lactose. Perkins utilized the following: the monosaccharids — dextrose and levulose; the disaccharids — lactose, maltose, and saccharose, the pentose arabinose, the triatomic alcohol glycerin and the hexatomic alcohol mannite, eight carbohydrates in all. In this work we increased the number of carbohydrates and alcohols to seventeen and we also used the protein sodium caseinate or nutrose.

We have utilized the monosaccharids—glucose, levulose, and galactose; the disaccharids—lactose, maltose, saccharose; the trisaccharids—raffinose; the polysaccharids—dextrin and inulin, the glucoside salicin, the pentoses arabinose and rhamnose, the hexahydric alcohols mannite, sorbite and dulcite, the pentahydric alcohol adonite, the trihydric alcohol glycerin, and the protein sodium caseinate. The method of procedure has been as follows: Each microorganism has been tested to determine whether or not it would ferment each of these c.p. carbohydrates, alcohols, and the protein, using litmus and phenolphthalein as indicators. With the latter the change in the reaction of the given medium could be determined, in each instance, by using an uninoculated tube as a control. With both indicators any evidence of gas formation in the fermentation tubes was recorded. The sugar broths were prepared according to the standard methods of the laboratory section of the American Public Health Association, using 1 percent of each substance. It may be said at once that nutrose (sodium caseinate) has been of no value whatever, and no further reference need be made to this substance here.

Titrations were made after four days at 37 C., with phenolphthalein as indicator; parallel observations were made at the same time with litmus as indicator and any evidence of gas formation recorded.

An analysis of these fermentation reactions shows that it is quite impossible to divide this group into species by means of differences shown in the reaction in the various carbohydrates and alcohols. Table 1 shows that in each instance some of the members of each group ferment one or more of the carbohydrates and alcohols of the different classes. Furthermore it will be observed that the more complex carbohydrate molecule is just as likely to be attacked as is a simpler one. This is not in accord with the findings of Howe,[13] Winslow[14] and others who have made biometrical studies of other groups of species. Where there is an increased acidity, with none in the control, or where blue litmus has changed to red, or where gas has been observed it is taken for granted that the carbohydrate has been fermented or split up. We believe that the present division of encapsulated gram-negative bacilli into various species here enumerated is not justified on the basis of their fermentation reactions. In connection with the fermentation reactions we have found eight members of this group of fourteen investigated, including bacillus capsulatus Pfeiffer, bacillus rhinoscleromatis, bacillus pneumonia Friedländer and bacillus sputigenus, all of which give gas formation in lactose bile. As this medium is used as a presumptive test for the presence of the bacillus coli in the examination of water samples, where sewage pollution is suspected, these results are of interest as they show that the statement of the committee of the Laboratory Section of the American Public Health Association, 1912, may require revision.

We have been able in this work to show in three experiments the presence of something in the Berkefeld filtrate of broth cultures of the bacillus capsulatus mucosus which, injected intravenously into guinea-pigs, caused death in less than twenty-four hours. This may be a diffusable or soluble toxin since the heart's blood culture in each instance was quite sterile, as was also the Berkefeld filtrate. This finding, which is in accord with that of Babes[15] and of Pawlowsky,[16] with the rhinoscleroma bacillus has no value in the differentiation of members of this group.

Our results with reference to the pathogenicity of the members of this group are in accord with those of Perkins, and are not satisfactory as a basis for classification. We have used guinea-pigs and rabbits and have found that cultures designated as *B. rhinoscleromatis,*

13. *Science, N. S.,* 1912, 35, p. 225.
14. *Jour. Infect. Dis.,* 1912, 10, p. 285.
15. *Handb. d. path. Mikroorg.,* 1913, 3, p. 436.
16. *Deutsch. med. Wchnschr.,* 1894, 14, p. 303.

TABLE 1

FERMENTATION REACTIONS OF THE MUCOSUS CAPSULATUS GROUP

	14 Cultures B. capsulatus mucosus	7 Cultures B. pneumoniae	5 Cultures B. rhinoscleromatis	5 Cultures B. capsulatus Pfeiffer	6 Cultures B. aërogenes	4 Cultures B. ozenae	1 Culture crassus sputigenus
Arabinose	11 of 14 +	3 of 4 +	4 of 5 +	4 of 4 +	4 of 4 +	3 of 4 +	I +
Rhamnose	11 of 14 +	3 of 4 +	4 of 5 +	4 of 4 +	4 of 4 +	2 of 4 +	I +
Dextrose	14 of 14 +	7 of 7 +	5 or 5 +	5 of 5 +	6 of 6 +	4 of 4 +	I +
Galactose	13 of 14 +	4 of 4 +	5 or 5 +	4 of 4 +	4 of 4 +	3 of 4 +	I +
Levulose	14 of 14 +	3 of 3 +	5 or 5 +	4 of 4 +	3 of 4 +	4 of 4 +	I +
Lactose	13 of 14 +	6 of 7 +	5 or 5 +	5 of 5 +	6 of 6 +	3 of 4 +	I +
Maltose	14 of 14 +	5 of 7 +	5 or 5 +	5 of 5 +	6 of 6 +	2 of 4 +	I +
Saccharose	12 of 14 +	7 of 7 +	5 or 5 +	5 of 5 +	6 of 6 +	2 of 4 +	I +
Dextrin	12 of 14 +	5 of 7 +	3 of 5 +	4 of 5 +	5 of 6 +	2 of 4 +	I +
Inulin	8 of 14 +	4 of 7 +	3 of 5 +	5 of 5 +	5 of 6 +	1 of 4 +	I +
Raffinose	11 of 14 +	5 of 7 +	5 of 5 +	5 of 5 +	6 of 6 +	2 of 4 +	I +
Adonite	11 of 14 +	4 of 5 +	3 of 5 +	5 of 5 +	5 of 6 +	2 of 4 +	I +
Dulcite	All negative	2 of 7 +	3 of 5 +	5 all negative	2 of 6 +	All negative	I negative
Mannite	13 of 14 +	6 of 7 +	4 of 5 +	5 of 5 +	6 of 6 +	2 of 4 +	I +
Sorbite	13 of 14 +	4 of 5 +	5 of 6 +	4 of 4 +	5 of 5 +	2 of 4 +	I +
Glycerin	13 of 14 +	3 of 4 +	5 of 5 +	4 of 4 +	4 of 4 +	3 of 4 +	I +
Salicin	9 of 14 +	5 of 7 +	5 of 5 +	5 of 5 +	6 of 6 +	2 of 4 +	I

+ = splitting of carbohydrate with acid or gas production.

B. capsulatus mucosus, B. pneumoniae Friedländer, etc., gave similar results when intraperitoneal injections were given. Some of the cultures had lost their virulence, but this was not characteristic of any one group.

The agglutination reaction and the reaction of fixation were employed in an endeavor to find a satisfactory basis of classification with results, which so far, are of limited value.

Beham and we have shown that members of this group are not agglutinable until they have lost their capsule. But they may act as antigens and produce agglutinins while still possessing a capsule. After they have lost their capsules they are agglutinable by appropriate sera. So far our results may be summarized as follows:

Cultures from various sources designated as *B. rhinoscleromatis* were agglutinated with immune rabbit serum. Unfortunately cultures with other designations possessed capsules while those of the cultures of the rhinoscleroma bacillus have been lost, so that differentiation was impossible. Further work in this direction will be undertaken. It may be said at this juncture, however, that if only those strains which have lost their capsules are agglutinable, it is obvious that with the great majority of freshly isolated cultures the reaction of agglutination will have no value as a means of differentiation. The acid agglutination reaction of Michaelis as modified by Beniasch[17] did not cause agglutination of the bacterium of rhinoscleroma without a capsule, or bacillus mucosus with a capsule.

The reaction of fixation has been tried with indifferent success. Antigens employed were first, broth cultures of the different strains but later antigens prepared according to the method used by Claypole[18] in her work with the streptothrix group was also tried. Rabbits were immunized with different strains and their sera with the different antigens and guinea-pig alexin was used. Cross fixations occurred even with quite low dilutions and very considerable anticomplementary activity was observed. While satisfactory fixation reactions were obtained, the homologous antigens and antisera gave no more satisfactory results than the heterologous. We were unable to obtain satisfactory results in the differentiation of Friedländer bacillus and the rhinoscleroma bacillus, by means of the reaction of fixation. We have so far been unable to obtain similar result.

17. *Ztschr. f. Immunitätsf.*, 1912, 12, p. 268.
18. *Jour. Exper. Med.*, 1913, 17, p. 99.

SUMMARY

So far our work cannot be taken to support any grouping of the gram-negative encapsulated bacilli heretofore proposed. Such divisions have been based on differences in staining reactions, cultural features, biochemical activities, or pathogenicity. After a careful review of these points in the cultures studied by us, it does not seem possible, at the present time, to constitute species on the basis of differences shown. It seems more than likely that this group is most closely related to the colon, the essential point of distinction being the possession of a capsule. It is conceivable that mutations, based on the necessity of maintaining a parasitic existence, have caused gram-negative bacilli, found normally in the body elsewhere than in the intestinal tract, to develop capsules for protection and a new group has arisen which we designate *B. capsulatus mucosus* and the varieties *B. aërogenes* and *B. acidi lactici* connect the group with the non-encapsulated gram-negative bacilli belonging to the colon group. This is not offered as a conclusion arising from the results here presented, but rather as a tentative suggestion.